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Kinetic Studies of Lipid Exchanges in Red Cell Membranes in Hereditary High Red Cell Membrane Phosphatidylcholine Hemolytic Anemia": Reference to the Abnormal Accumulation of Phosphatidylcholine in These Membranes

Akiyo OTSUKA, Takashi SUGIHARA and Yoshihito YAWATA

Department of Medicine, Kawasaki Medical School
Kurashiki 701-01, Japan

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ABSTRACT. The pathogenesis of the accumulation of phosphatidylcholine (PC) in red cell membranes of the patients with hereditary high red cell membrane phosphatidylcholine hemolytic anemia (HPCHA) was studied utilizing ^{14}C-PC and ^{14}C-lyso-PC.

The uptake of ^{14}C-PC was not significantly different in the red cell membranes with HPCHA from that in normal controls and obstructive jaundice in whom red cell membrane PC and free cholesterol (FC) were elevated to the same extent as that in HPCHA. The efflux of ^{14}C-PC with HPCHA was slightly decreased, in comparison with that in the normal controls and obstructive jaundice. The most striking data were obtained in the uptake of ^{14}C-lyso-PC by the red cells of the HPCHA patients, showing more than two fold increase when compared with that in the normal control. The conversion of ^{14}C-lyso-PC to ^{14}C-PC was increased in comparison with that in the normal controls and obstructive jaundice.

In summary, the exact cause of the abnormal accumulation of PC through increased uptake of lyso-PC, followed by the increased conversion of lyso-PC to PC is unknown. In obstructive jaundice of an acquired origin, some compensatory mechanism may exist to prevent the further accumulation of lipids in the red cell membranes.

Key words : hereditary high red cell membrane phosphatidylcholine hemolytic anemia (HPCHA) — phosphatidylcholine — lyso-phosphatidylcholine — obstructive jaundice

So-called "hereditary high red cell membrane phosphatidylcholine hemolytic anemia (HPCHA)" is characterized by hemolytic anemia of congenital origin with increased red cell membrane lipids, especially free cholesterol (FC) and phosphatidylcholine (PC), although plasma lipids in these patients are essentially normal. The first cases were reported by Jaffé *et al.* in 1968,[1] and since then several others have been described.[2–7] Mild hemolysis and the presence of stomatocytes (xerocytosis) and of target cells in peripheral blood are the clinical hallmarks of HPCHA.

In 1971, Shohet *et al.* studied the mechanism that leads to the accumulation of membrane lipids only in the red cells of HPCHA and suggested that the lipid exchange pathway from PC to phosphatidylethanolamine (PE) is defective.[5] The exact pathogenesis of the accumulation of membrane PC in

大塚明代，杉原　尚，八幡義人

HPCHA, however, has not been clearly elucidated. In this communication, 6 cases of HPCHA are introduced in an attempt to clarify the reasons for abnormal uptake of ^{14}C-PC and ^{14}C-lyso-PC. Results in the HPCHA patients were compared with those in 9 normal controls and 4 patients with obstructive jaundice in whom red cell membrane lipids were markedly increased concomitant to increased plasma lipids of acquired origin.

MATERIALS AND METHODS

Materials

Six patients with HPCHA and four patients with obstructive jaundice who showed a marked increase in red cell membrane lipids, were chosen for this study. Nine normal healthy subjects were selected as controls.

Venous blood was taken with heparin during a fasting state, and plasma and buffy coats were carefully removed by aspiration after centrifugation at 2500 g for 10 minutes. The resulting red cells were washed three times with five volumes of isotonic saline.

Extraction and analysis of red cell membrane lipids

Red cells were separated from plasma by centrifugation and washed three times with isotonic saline (pH 7.4). Red cell membrane lipids were extracted by Rose's method.[8] One ml of packed red cells was hemolyzed with 1 ml of distilled water, and then 11 ml of isopropylalcohol were added. After 1 hour, 7 ml of chloroform were added slowly using vortex mixer. Another 1 hour later, 4 ml of isotonic saline were added. The mixtures were kept at 4°C overnight, then they were centrifuged at 3000 g for 15 minutes, and the upper layer was discarded to remove water. The remaining lipid extracts were filtered and stored in a refrigerator at −70°C. Free cholesterol and phospholipids in the extracts were determined by the methods of Richmond[9] and Bartlett,[10] respectively. Phospholipids were separated and identified by thin-layer chromatography on Silica Gel (Merck) developed with a solvent system: chloroform-methanol-acetic acid-water (25:15:4:2).[11]

Uptake of phosphatidylcholine

Washed red cells were suspended in an incubation medium containing 0.154 M Na/K phosphate-buffered saline with 0.22 M glucose and 0.1% human albumin, and liposomes (a 1 to 1 ratio of phosphatidylcholine/free cholesterol), which were prepared by sonication with phosphatidylcholine, L-A-dipalmitoyl-^{14}C (New England Nuclear NEC-76410).[12,13] The cells were incubated at 37°C for 6 hours and then red cells were washed three times with the phosphate-buffered saline to extract red cell membrane lipids.[8] The radioactivities in whole red cells and in extracted lipids were determined by liquid scintillation counting.

Efflux of phosphatidylcholine

Washed red cells were suspended in the same incubation medium with the liposomes for 3 hours, after which they were washed three times with five volumes of phosphate-buffered saline to remove any phosphatidylcholine adhesing to the red cell membranes. Then the red cells were reincubated for

3 hours at 37°C in the incubation medium with the same liposomes, but with radioactive phosphatidylcholine being omitted. After the incubation, these cells were washed three times with phosphate-buffered saline and then the radioactivities of the whole red cells and extracted lipids were determined.[14] Red cell membrane lipid contents were also determined to calculate the specific activity.

Uptake of lyso-phosphatidylcholine

The incubation medium was prepared with lysopalmitoyl phosphatidylcholine, L-1-^{14}C (New England Nuclear: NEC-68310) and 3 mg/dl of L-α-lysophosphatidylcholine, palmitoyl (Sigma, Mo L5254) and contained 0.22 M glucose and 0.5% human albumin. Red cells were incubated in the lipid-containing medium for 6 hours at 37°C, after which they were washed with a phosphate buffer contained 1% bovine albumin (Sigma, Mo A6793) to remove the labile lyso-phosphatidylcholine on the red cell membranes. Membrane lipids were extracted for radiochemical and biochemical analysis.[15,16]

A portion of the extracted red cell membrane lipids was applied to silica gel plates to separate each phospholipid fraction by thin-layer chromatography using a solvent system of chloroform: methanol: acetic acid: water (25:15:4:2). Phosphate determinations were performed on the fractionated phospholipids, and radioactivities were counted by liquid scintillation counting.

RESULTS

Hematological findings in the 6 patients with HPCHA are shown in Table 1. Two of these patients were unsplenectomized, while the other four had been splenectomized at least 6 months earlier.

The splenectomy per se did not affect the clinical pictures, including the extent of anemia, reticulocyte counts, or the concentration of indirect bilirubin, as previously described at our laboratory.[17]

The diagnosis of HPCHA was made, as based on the criteria of Jaffé *et al.*[1] i.e., of congenital origin and characterized by moderate uncompensated hemolytic anemia, increased levels of red cell membrane PC but normal levels of other phospholipids, normal plasma lipid levels, normal activity of lecithin: cholesterol acyl transferase, increased sodium transport, and others.

TABLE 1. Hematological findings of HPCHA

	Sex male / female	RBC ($\times 10^6/\mu l$)	Hb (g/dl)	MCV (fl)	MCH (pg)	MCHC (%)	Reticulocytes (%)	Indirect bilirubin (mg/dl)
Normal (n=9)	7 / 2	4.48 ±0.69	14.2 ±2.0	94.4 ±8.0	31.8 ±3.0	34.1 ±1.5	1.0 ±0.5	0.4 ±0.2
HPCHA (n=6)	2 / 4	2.40 ±0.30	9.2 ±0.7	107.5 ±5.2	38.0 ±1.9	35.5 ±0.	8.7 ±5.7	3.1 ±1.4

TABLE 2. Red cell membrane lipids in the patients with HPCHA and obstructive jaundice

	FC	PL	PE	PS+PI	PC	SM	L-PC
	(μg/10^{10} RBC)						
Normal (n=9)	1200 ±101	2609 ±240	804 ±85	365 ±38	731 ±62	662 ±73	38 ±10
HPCHA (n=6)	1856 ±256	3483 ±419	898 ±116	522 ±90	1143 ±178	785 ±108	53 ±32
Obstructive jaundice (n=4)	1679 ±472	2977 ±469	687 ±55	415 ±36	1068 ±318	738 ±127	64 ±34
Statistical significance between the groups shown above (P values)							
*	P<0.001	P<0.001	N.S.	P<0.01	P<0.001	P<0.01	P<0.01
†	N.S.	N.S.	N.S.	N.S.	P<0.1	P<0.05	P<0.02
‡	N.S.	N.S.	N.S.	N.S.	N.S.	P<0.05	P<0.01

N.S. : not significant

FC: free cholesterol PL: phospholipids PE: phosphatidylethanolamine
PS: phosphatidylserine PI: phosphatidylinositol PC: phosphatidylcholine
SM: sphingomyelin L-PC: lyso-PC
* Normal — HPCHA † Normal — Obstructive jaundice
‡ HPCHA — Obstructive jaundice

Red cell membrane lipids

The lipid contents of the red cell membranes from the patients with HPCHA, obstructive jaundice and normal controls are shown in Table 2. In the HPCHA patients, FC and total phospholipid contents were clearly elevated as compared with those in normal controls ($P<0.001$). Among phospholipids, the increase of PC was most marked, accompanied by slight increase in phosphatidylserine (PS), phosphatidylinositol (PI), sphingomyelin (SM) and lyso-PC. In the obstructive jaundice patients, FC and PC had also increased to almost the same extent as observed in the HPCHA cases.

Uptake of phosphatidylcholine

Red cells were incubated in the medium containing radioactive PC at 37°C for 6 hours. The amounts of PC incorporated into red cells are shown in Fig. 1. No significant differences were observed between the HPCHA patients (89.1 ± 34.5 n moles/10^{10} RBC/6 hours) and normal controls (81.4 ± 11.4) although the membrane lipid contents were already elevated in the HPCHA patients. In contrast, the uptake of PC was rather decreased in the obstructive jaundice patients (51.2 ± 8.7, $P<0.001$), in whom red cell FC and PC were markedly increased in the same manner as in the HPCHA. This may indicate that, in obstructive jaundice, a compensatory mechanism to prevent further accumulation of PC in red cells may exist.

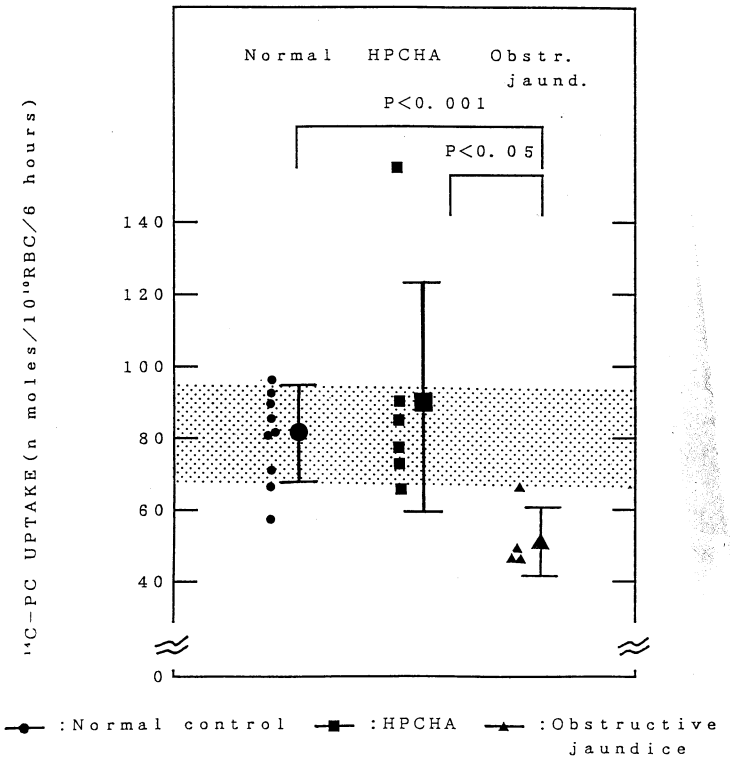

Fig. 1. The amounts of uptake of PC in red cells with normal control, the patients of HPCHA and obstructive jaundice. The red cells were incubated in ^{14}C-phosphatidylcholine liposomes (free cholesterol: phosphatidylcholine=1:1) at 37°C for 6 hours. After washing the red cells incubated, the amounts of uptake of PC in red cells were calculated, as based on the specific activity remained in the red cells. Closed circles denote normal control, closed squares depict HPCHA, and closed triangles show obstructive jaundice.

Efflux of phosphatidylcholine

The efflux of PC was calculated by determining the radioactivity of the PC remaining in red cells after the further incubation with unlabeled PC. The ratio of the amount of PC eluted from the red cells to that of PC incorporated initially into the red cells is shown in Fig. 2. The amount of PC eluted from red cells was much less in HPCHA cells (32.5±16.8%) than the amounts in the normal controls (49.8±8.1, $P<0.05$) and the obstructive jaundice (72.3±31.3, $P<0.05$) in whom red cell FC and PC were markedly elevated.

Uptake of lyso-phosphatidylcholine

The amounts of lyso-PC uptake are shown in Fig. 3. A marked increase in lyso-PC uptake was observed in HPCHA red cells (61.6±30.2 n moles/10^{10} RBC/6 hours, $P<0.05$). In obstructive jaundice patients, on the other hand, lyso-PC uptake was rather decreased (21.6±9.8, $P<0.02$), compared to that in HPCHA.

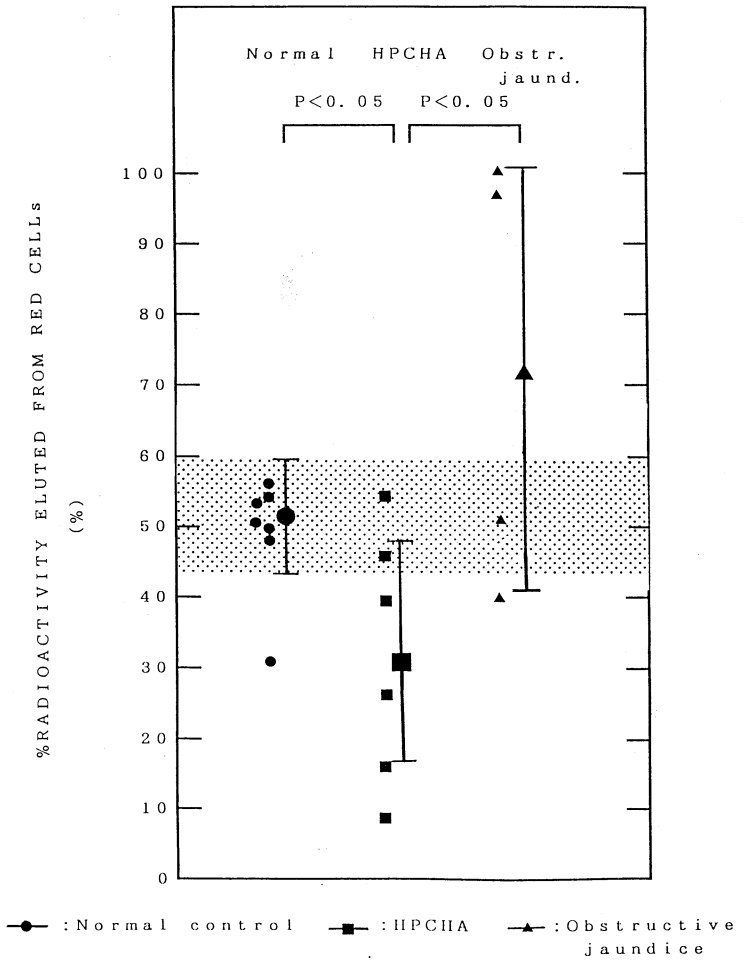

Fig. 2. The efflux of PC from red cells with normal control, HPCHA and obstructive jaundice. The symbols are the same as shown in Fig. 1. Red cells of normal control, HPCHA and obstructive jaundice were preincubated with ^{14}C-PC (see the detail of the experimental conditions in text), and after washing the red cells were further incubated in the incubation medium without radioactive PC. The ratio of PC eluted from the red cells to incorporated PC were calculated from the specific activities.

Conversion of lyso-PC to PC

After incubation of red cells with lyso-PC, ^{14}C radioactivity was found to be present not only in lyso-PC, but also in PC, as detected by liquid scintillation counting on thin-layer chromatography. The ratio of conversion from lyso-PC to PC is shown in Fig. 4. In HPCHA, the ratio of PC to incorporated lyso-PC was elevated (0.73 ± 0.06) than normal controls (0.62 ± 0.14, $P<0.1$), and obstructive jaundice (0.59 ± 0.02, $P<0.02$).

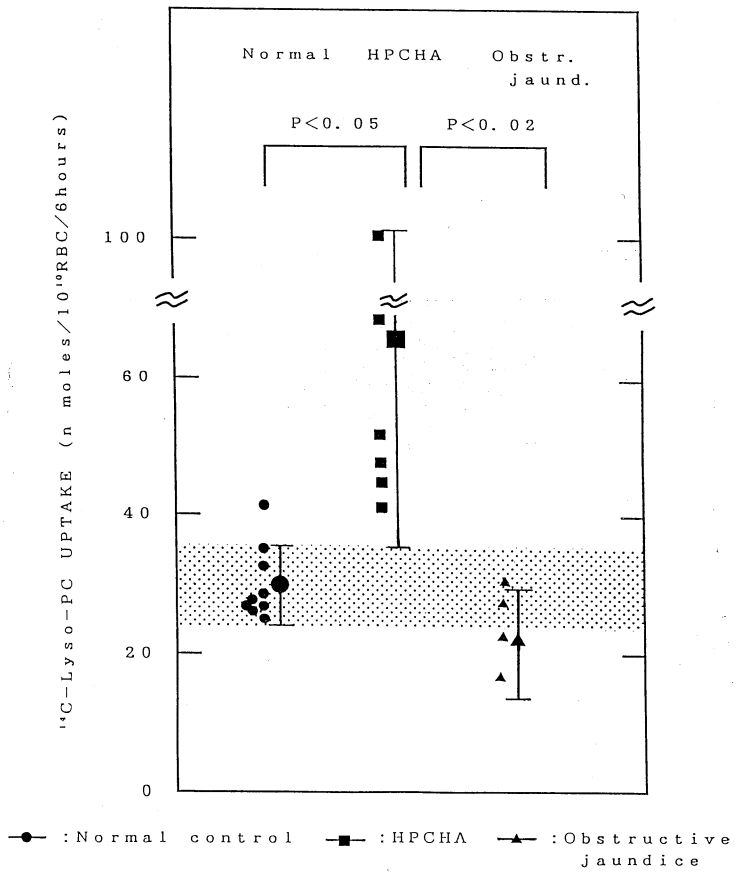

Fig. 3. The amounts of uptake of lyso-PC in red cells of normal control, HPCHA, and obstructive jaundice. The red cells were incubated with the incubation medium with ^{14}C-lyso-PC at 37°C for 6 hours. The amounts of uptake of lyso-PC were calculated from the specific activities in these red cells. The symbols are the same as shown in Fig. 1.

DISCUSSION

Since human mature red cells cannot synthesize membrane lipids, red cell membrane lipids should be constructed essentially by utilizing plasma lipids.[18] In hereditary spherocytosis (HS), for example, the level of red cell membrane lipids is lower in association with decreased plasma lipids.[19–21] On the other hand, in hepatic diseases, such as obsrtuctive jaundice, a marked increase in membrane lipids has been observed in association with increased plasma lipids.[22,23] These observation may indicate the presence of a metabolic relationship between red cell membrane lipids and plasma lipids.

In HPCHA, red cell membrane lipids (especially FC and PC) have been observed to increase markedly, although the levels of plasma lipids are essentially normal.[24] It has thus been suggested that the abnormality in red cell membrane lipids in HPCHA is not due to abnormal plasma lipids. The exact

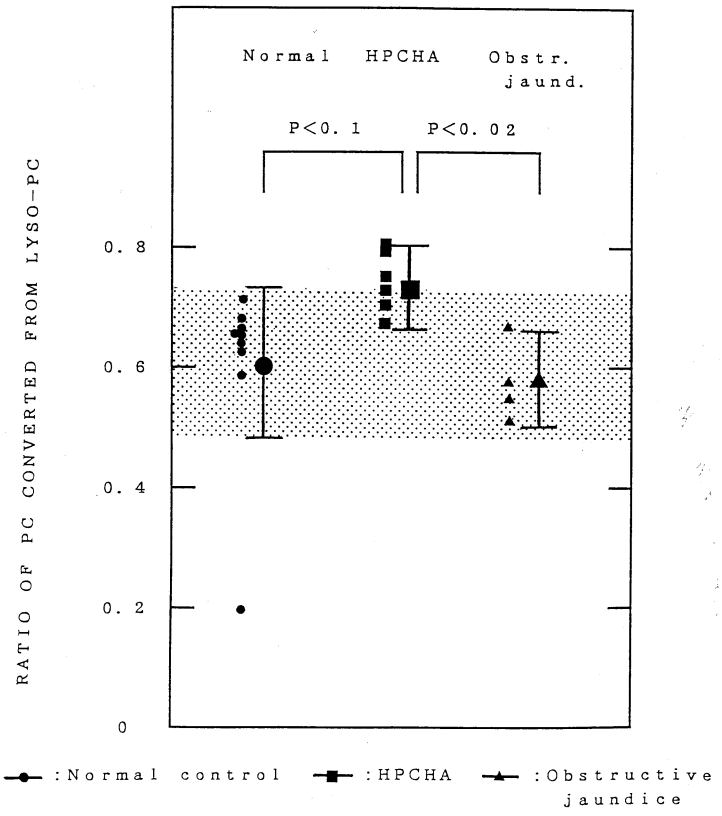

Fig. 4. The ratio of PC converted from lyso-PC to incorporated lyso-PC into the red cells with normal control, HPCHA and obstructive jaundice. The red cells were incubated with ^{14}C-lyso-PC at 37°C for 6 hours. After the incubation, radioactivities were determined at the spots as PC and lyso-PC on thin-layer chromatography. The ratio of the amounts of PC, which was converted from lyso-PC during incubation, to the amounts of lyso-PC, which was initially incorporated during the first incubation.

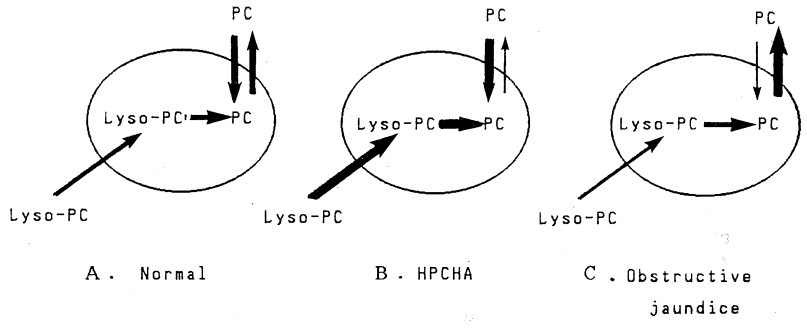

Fig. 5. Schematic diagrams on kinetics of PC and lyso-PC in the red cells of normal control (A), HPCHA (B), and obstructive jaundice (C).

mechanism for accumulation of FC and PC in red cell membranes may exist in an abnormality in the regulation of lipid renewal. In this study, the red cells in HPCHA demonstrated a markedly increased lyso-PC uptake, and a significant increase of lyso-PC conversion of which incorporated to PC. In the dynamic movement of PC from the lipid medium to red cell membranes, however, uptake was not inhibited in HPCHA cells in spite of the high level of red cell membrane lipids.

Based on these results, these two disorders with membrane lipid abnormalities, HPCHA and obstructive jaundice, can be consider to be quite different in their lipid kinetics. In HPCHA, the increased uptake of lyso-PC and its enhanced conversion to PC in the red cell membranes appear to be the most important factors, in the pathogenesis of HPCHA. The results of this study are schematically shown in Fig. 5.

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